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Calumenin has a role in the alleviation of ER stress in neonatal rat cardiomyocytes

Joo Hee Lee, Eun Jeong Kwon, Do Han Kim *

School of Life Sciences and Systems Biology Research Center, Gwangju Institute of Science and Technology (GIST), 123 Cheomdan-gwagiro (1 Oryong-dong), Buk-gu, Gwangju 500-712, Republic of Korea

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ABSTRACT

Disturbance of endoplasmic reticulum (ER) homeostasis causes ER stress (ERS), and triggers the unfolded protein response (UPR) that consequently reduces accumulation of unfolded proteins by increasing the quantity of ER chaperones. Calumenin, a Ca^{2+}-binding protein with multiple EF hand motifs, which is located in the ER/SR, is highly expressed during the early developmental stage of the heart, similar to other ER-resident chaperones. The aim of this study was to investigate the functional role of calumenin during ERS in the heart. Like other chaperones (e.g., GRP94 and GRP78), calumenin expression was highly upregulated during ERS induced by 10 μg/ml tunicamycin, but attenuated in the presence of 500 μM PBA, the chemical chaperone in neonatal rat ventricular cardiomyocytes (NRVCs). Upon 7.5-fold overexpression of calumenin using a recombinant adenovirus system, the expression levels of ERS markers (GRP78, p-PERK, and p-eIF2α) and ER-initiated apoptosis markers (CHOP and p-JNK) were reduced, whereas the survival protein BCL-2 was upregulated during ERS compared to the control. Evaluation of cell viability by TUNEL assay showed that apoptosis was also significantly reduced by calumenin overexpression in ERS-induced cells. Taken together, our results suggest that calumenin plays an essential role in the alleviation of ERS in neonatal rat cardiomyocytes.

1. Introduction

The endoplasmic reticulum (ER) is an intracellular organelle involved in multiple cellular processes such as protein synthesis, lipid biosynthesis, protein folding, posttranslational modification, transport of proteins, and regulation of calcium homeostasis. Perturbation of ER function, which is caused by various factors, such as ischemia, hypoxia, exposure to free radicals, elevated protein synthesis, and gene mutation, can lead to accumulation of unfolded and misfolded proteins in the ER, a condition referred to as ER stress (ERS) [1–3]. ERS induces the unfolded protein response (UPR), an adaptive response to restore ER homeostasis by reducing the accumulation of unfolded proteins. When ERS occurs, the ER molecular chaperone Bip/GRP78 dissociates from the three ER trans-membrane sensors—protein kinase-like ER kinase (PERK), inositol requiring kinase 1 (IRE1), and activating transcription factor 6 (ATF6)—leading to their activation. Activation of UPR results in translational inhibition, thereby reducing the production of new proteins in ER, transcriptional activation of genes for chaperones and folding enzyme, and promotion of ER-associated degradation (ERAD) to remove misfolded proteins. In the heart, ER chaperones such as GRP94, GRP78, and calreticulin are upregulated in response to UPR to enhance the ability of the SR to regulate its intracellular Ca^{2+} level and to reduce the quantity of unfolded proteins by supporting their proper folding, a cardioprotective effect [4–7].

Nevertheless, if the stress is severe or prolonged, the UPR fails to protect cells against ERS. The signaling switches from pro-survival (adaptive response) to pro-apoptosis (maladaptive response) by transcriptional induction of C/EBP homologous protein (CHOP) or by the activation of c-Jun N-terminal kinase (JNK) and caspase-12–dependent pathways [8,9]. Recently, the UPR and ER-initiated apoptosis have been implicated in the progression of cardiovascular diseases such as ischemic and non-ischemic heart failures [4,7,10–12]. Therefore, pathways for coping with ERS may be an important target for the development of therapeutic approaches against cardiovascular diseases.

Abbreviations: ATF, activating transcription factor; Ad-calu, adenovirus containing mouse calumenin 1 cDNA; Ad-lacZ, adenovirus containing lacZ gene; CHOP, C/EBP-homologous protein; ERAD, ER-associated degradation; eIF2, eukaryotic initiation factor 2; ERp57, the thiol oxidoreductases proteins disulphide isomerase and ER protein; GRP, glucose related protein; ifu, infectious unit; IRE, inositol-requiring enzyme; MOI, multiplicity of infection; NRVCs, neonatal rat ventricular cardiomyocytes; PERK, protein kinase RNA-like endoplasmic reticulum kinase; PBA, 4-phenylbutyric acid; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Tg, thapsigargin; TM, tunicamycin; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; UPR, unfolded protein response.

* Corresponding author. Fax: +82 62 970 3411.
E-mail address: dhkim@gist.ac.kr (D.H. Kim).

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Calumenin is a Ca^{2+}-binding protein located in the SR lumen of mammalian heart and belongs to the CREC family of Ca^{2+}-binding proteins having multiple EF-hand domains [13]. Calumenin is ubiquitously expressed, albeit at a higher expression level in the heart. It has also been reported that the mRNA expression level of calumenin in embryonic heart is higher than that in adult mouse heart [14]. Likewise, calumenin protein expression markedly decreases and achieves a steady state in adult mouse heart compared to embryonic and neonatal heart [15]. Similarly, the expression pattern of several ER resident chaperones such as calreticulin, GRP78, GRP94, protein disulfide isomerase (PDI) and ER protein 57 (ERp57) were found to be highly expressed during earlier stages of development [16]. It has been observed that overexpression of ER-resident molecular chaperones, such as GRP78 and GRP94, can protect cells from cell death by attenuating ERS [17–19]. Further studies have shown that chemical chaperones can attenuate the progression of cardiovascular diseases [10,20]. Thus, this study is interested in exploring whether calumenin, a multifunctional CREC family protein, has a chaperone-like role in the heart.

In the present study, we explored the possible chaperone-like role of calumenin for ERS and ER-initiated apoptosis using the recombinant adenovirus overexpression system in neonatal rat ventricular cardiomyocytes (NRVCs). The results showed that overexpression of calumenin significantly alleviated ERS and inhibited ER-initiated cellular apoptosis, suggesting that calumenin may be an important molecular chaperone in NRVCs.

2. Materials and methods

2.1. Preparation of neonatal rat ventricular cardiomyocytes

Neonatal rat ventricular cardiomyocytes (NRVCs) were isolated from 2-day-old neonatal Sprague–Dawley rats using the Neonatal Cardiomyocyte Isolation System (Worthington Biochemical Corporation, Lakewood, NJ, USA). The aortic root and atria were removed prior to isolation. Isolated cells were plated in gelatin-coated culture dishes. The number of cells was approximately 1×10^6 cells per 60-mm dish. Cells were suspended in DMEM (low glucose) with 10% fetal bovine serum (FBS), 1% antibiotics, and 0.1 mM BrdU, followed by incubation with 5% CO_2 and 98% relative humidity at 37 °C in a culture incubator. All animal procedures were approved by the GIST Institutional Animal Care and Use Committee.

2.2. Recombinant adenovirus amplification

Previously generated recombinant adenovirus containing mouse calumenin-1 cDNA (Ad-calu) [21] was amplified in HEK 293 cells. Adenovirus containing the lacZ gene (Ad-lacZ) was used as a control. The infectious unit (ifu) of amplified viruses was determined by titration using the Adeno-X rapid titer kit purchased from Clontech Laboratories (Mountain View, CA, USA). Various multiplicities of infection (MOIs) of viruses were used for generating calumenin overexpression in NRVCs.

2.3. Adenoviral infection of NRVCs and treatment of the infected cells with reagents to trigger ERS

After a 24 h incubation period of NRVCs in the low glucose DMEM media (as shown in Section 2.1), the media were replaced with a serum (FBS)-free media to starve the cells. During the starvation period, the recombinant adenoviruses were infected for 6 h followed by a 1× change into serum-free media. After an additional 12 h maintenance period, 10 μg/ml TM or 2 μM Tg was treated for 12 h to induce ERS. To observe the effect of a chemical chaperone, 500 μM PBA was treated in the presence or absence of 10 μg/ml TM and incubated for 24 h.

2.4. Western blot analysis

To check the expression levels of proteins, Western blot analysis was performed. Cells were lysed in 100 μL SDS lysis buffer (1% sodium dodecyl sulfate, 10 mM Tris–HCl with protease inhibitor cocktail). The BCA protein assay kit (Pierce, Rockford, IL, USA) was used for protein quantification. Cardiomyocyte lysate was solubilized in 5× sample buffer containing β-mercaptoethanol, and the constituent proteins were separated by SDS–PAGE and transferred to polyvinylidene fluoride (PVDF) membrane. The membranes were then incubated with 5% skim milk or 5% BSA in Tris–HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20 (TBST) for 1 h at room temperature to block non-specific binding of antibody. Next, the membranes were incubated overnight at 4 °C with primary antibodies against one of the following proteins: calumenin, KDEL (GRP78, 94), p-PERK, PERK, p-eIF2α, CHOP, Bcl-2, p-JNK, JNK, α-tubulin, and GAPDH. After primary antibody incubation, membranes were washed with TBST and further incubated with the appropriate peroxidase-conjugated secondary antibody. Western blot band signals were detected by using the Image Quant Las 4000 mini (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and SuperSignal West Pico Chemiluminescence Kit (Thermo Fisher Scientific, Inc., Waltham, MA). Band intensities were measured by ImageJ software (NIH).

2.5. TUNEL assay

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays were performed to detect and quantify apoptotic cell death at the single cell level. NRVCs were plated on gelatin-coated cover slips, incubated and infected by recombinant adenoviruses at MOI 50, and treated with 10 μg/ml TM. After 12 h of TM treatment, cells were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 1 h at room temperature. The slides were washed with PBS and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. Nuclear staining was accomplished by Hoechst 33342, and apoptotic cells were labeled by a TUNEL reaction mixture containing terminal deoxynucleotidyl transferase using In Situ Cell Death Detection Kit (Roche Applied Science, Penzberg, Germany). After incubation for 1 h at 37 °C, the stained slides were washed 3 times with PBS and observed under a Olympus confocal microscope FV1000 (Olympus, Tokyo, Japan). The number of TUNEL-positive cells was measured using Metamorph software.

2.6. Statistical analysis

The experimental values are represented as mean ± S.E.M. Multiple comparisons between the different types of samples were conducted using an ANOVA test. A value of $P < 0.05$ was used as the criterion for identifying statistically significant differences.

3. Results

3.1. Calumenin was upregulated by ER stress in NRVCs

In order to investigate whether calumenin is involved in the alleviation of ERS in NRVCs, the cells were treated with 10 μg/ml tunicamycin (TM) and the expression levels of calumenin were examined. Calumenin was upregulated by ERS in a manner similar to GRP78, the marker protein of ERS (Fig. 1). The downward shift in molecular weight of calumenin after addition of TM may be due to the deglycosylation of calumenin, since TM blocks the synthesis of